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# REPRODUCTIVE SUCCESS IN HATCHERY-PRODUCED EASTERN OYSTER, CRASSOSTREA VIRGINICA (GMELIN)

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# REPRODUCTIVE SUCCESS IN HATCHERY-PRODUCED EASTERN OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN)

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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## TABLE OF CONTENTS

Page

## List of Tables

Page

## List of Figures

Page

**Abstract**

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The eastern oyster (*Crassostrea virginica* Gmelin 1791) has great ecological and economic importance but populations have declined, especially in Chesapeake Bay, to historically low numbers. Hatcheries strive to produce oysters with beneficial characteristics for supplementation and commercial purposes, both natural and stimulated mass spawning. Unequal contribution of parents in mass spawnings potentially can lead to high levels of inbreeding and a loss of beneficial characteristics in offspring. In this study, we determined microsatellite genotypes for parents ($n_{parents}$ =24, 49, and 77 parents) and progeny (n=96 each) of three hatchery-produced families and used the data for parental assignment. We observed the presence of more than two alleles per locus for some offspring, yet because genetic analysis software only allows for a

maximum of two alleles per locus, we chose the two alleles with the strongest signals. For the three parent "populations," 71% of alleles had frequencies of <0.05 and observed heterozygosities were lower than expected by an average factor of 0.27. Inbreeding within the various parent populations was similar across the three families ranging from $F_{IS}$ 0.26–0.43. In all three families, the offspring exhibited greater levels of genetic diversity and lower inbreeding levels than the parents ($F_{IS}$ 0.14–0.21), and in some cases offspring exhibited alleles that were not present in the parents. Variance in the number of offspring produced per parent was observed for all families and in general, <10% of potential parents (generally 2-5 females and 1-3 males) produced >10% of the offspring. Reproductive success for spawning parents, $N_b$, determined by three methods, ranged from 0.07 to 0.27. As the number of parents per family increased, a higher proportion of parents failed to produce offspring. Across all three families, the average effective number of breeders was $N_b$ = 7.1 and the level of reproductive success was inversely proportional to the number of potential parents. This finding implies that to maintain high levels of diversity and beneficial characteristics in the offspring (and to avoid the chance of unintentional inbreeding), hatcheries should perform more spawnings with fewer parents.

# INTRODUCTION

The eastern oyster (*Crassostrea virginica*) is an important ecological component of estuarine habitats and is considered a non-polluting aquaculture commodity (Lenihan and Peterson 1998). Oysters are suspension feeders noted in restoration and ecology for their role in passing nutrients and organic matter from pelagic to benthic food webs (Newell 1988). Oysters flourish when they live in a combination of banks and beds (an oyster reef), which generally occur in shallow waters. However, many formerly productive reefs worldwide have been completely removed (Hargis and Haven 1999); in particular, the mechanical destruction of Chesapeake Bay oyster beds via oyster dredges has reduced the total oyster habitat to less than 50% of what it was in the 1800s (Rothschild et al. 1994). Because of excessive harvesting and habitat loss, oyster populations along the US Atlantic coast have been dramatically reduced. In 1994, it was reported that the catch had declined from a peak of 615,000 metric tons in 1884 to 12,000 metric tons in 1992 (Rothschild et al. 1994). As of 2012, this has hardly changed, with a reported catch of 15008 metric tons (NOAA 2012). In addition to the effects of habitat destruction and overharvesting, Chesapeake Bay oysters continue to decline due to introduced protozoan parasites *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) (Dittman et al. 2001). Because of their ecological and economic potential for benefit, much effort is devoted to aquaculture production and rearing of the eastern oyster, including innumerable small-scale local oyster gardening endeavors and supplementation efforts supported by hatcheries, some rearing in

excess of $1\times10^9$ oysters per year. In 2013, $3\times10^7$ oysters aquacultured in the Chesapeake Bay were sold at market (VIMS Report 2014-5).

Successful oyster aquaculture and restoration are tied intimately to the identification of rapid growing, disease tolerant oyster strains. For decades, researchers conducted trials to obtain oysters that both grow rapidly and tolerate the parasites, Dermo and MSX. Efforts have met with mixed success, partly due to lack of a means of selecting appropriate broodstock (Calvo et al. 2002; Rawson and Feindel 2012). In the Chesapeake Bay, hatchery production of oysters is a primary means of producing disease-free spat (juvenile oysters) for supplementation, oyster gardening, and commercial aquaculture. Producing spat from oyster strains that grow rapidly, survive disease stress, or have other beneficial production characteristics such as aesthetically appealing shell shape has tremendous value.

In the hatchery, conditioned oysters (Loosanoff and Davis 1963, Hidu et al. 1969) are spawned beginning in the early spring. Individual oysters are placed on a table with running water that is slowly warmed to 20-25°C, generally about 5°C above ambient temperature. This warmer temperature should cause the oysters to begin spawning *en masse* (natural spawning) but if they do not respond, some oysters may be sacrificed so their sperm or eggs can be mechanically stripped from the gonad and pipetted into a reduced volume of water surrounding other oysters to induce the remaining oysters to spawn (stimulated spawning, Smith and Chanley 1975, Wallace 2008). Both techniques are regularly employed for the Eastern oyster and once spawning oysters are identified in the mass bath, they are removed and placed in a separate standing water bath for continued gamete release and fertilization.

Unequal contribution of parents in such mass spawnings has previously been identified as a potential issue for other oyster species reared in the hatchery (Lallias et al. 2010, Boudry et al. 2002). If not identified and controlled, such reproductive variance could result in higher than expected levels of inbreeding and possibly negative consequences for larval development or vigor that could be counterproductive for domestication and restoration efforts. For example, studies of the Pacific oyster, *Crassostrea gigas*, revealed high variance in family size at various stages of development that were attributed to quality of and interaction between gametes and differences in viability among the observed genotypes (Boudry et al. 2002). Mass selection lines and cultured populations of *C. gigas* also have shown lower than expected heterozygosity levels (Appleyard and Ward, 2006; Li et al. 2006), likely due to the presence of null alleles (Carlsson 2008). Lallias et al. (2010) noted that a single male generally contributed 50-100% of the progeny assayed for both wild and hatchery-produced families of another oyster species, *Ostrea edulis*. Similarly, work with other hatchery-produced species (e.g., American shad; Brown et al. 2000a) has indicated that there may be marked differences in the reproductive success of individual parents, especially if they are included in mass spawning events where the gametes of multiple males and females are mixed. The extent to which this phenomenon, termed "reproductive variance" or "reproductive success" occurs in hatcheries of *C. virginica* may affect production of spat with the intended growth, disease tolerance, and reproductive performance characteristics.

*Study objectives*

To assess reproductive success in an eastern oyster hatchery, this study used microsatellite markers from *C. virginica* to evaluate parents and progeny of three hatchery-produced oyster families. Two of the families were induced to naturally spawn and the third was stimulated by introducing sperm stripped from a male oyster to induce the other oysters into spawning (stimulated spawning).

## MATERIALS AND METHODS

*Hatchery produced oyster families*

Three mass-spawned families of oyster were produced at Horn Point Environmental Laboratory in Cambridge, MD in the summer of 2013 by collaborator Donald Meritt. All parents for the three spawnings were frozen in the shell immediately following the spawning. Sperm from the stripped male used in the stimulated-spawning family was not used to fertilize the eggs and was not genotyped or included in parentage analysis. Randomly collected 2-day old larvae (preserved in 70% EtOH) and 2-week old (preserved in RNAlater (Qiagen)) larvae were shipped to VCU. Of the larvae, 96 of the 2-week old were haphazardly selected for DNA preparation and genotyping.

*DNA preparation and genotyping using published loci*

Parental genomic DNA was prepared for PCR from *C. virginica* somatic tissue with the DirectAmp™ Tissue Genomic DNA Amplification Kit (MO BIO Laboratories, Inc). Larval genomic DNA was isolated using the QIAamp® DNA Mini Kit (QIAGEN). Samples were amplified separately for each of 12 previously published variable microsatellites (Table 1). One of each primer in a pair was modified to include a tail (Boutin-Ganache et al. 2001) allowing incorporation of a labeled third primer that facilitated pooling of six reactions for each individual, which greatly accelerated the automated genotyping. Each 10 μL PCR genotyping

reaction contained 5 µL GoTaq™ mastermix (Promega, Inc.), 1.0 µL primer mix (0.5 µM final concentration for tailed and reverse primers, 0.08 µM final concentration for forward primer), 1 µL DNA template, and 3.0 µL nuclease-free water. It was necessary for these DNA preparations to modify the published PCR protocols. The optimized procedure consisted of an initial denaturation for 2 min at 95°C, followed by 50 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s. Amplification products were resolved via ABI 3730XL and each individual's assignments were checked manually with GeneMarker version 2.6.2 (SoftGenetics, LLC, PA).

*Statistical Analyses*

Genotypic proportions, allele frequencies (total and effective), observed and expected heterozygosities ($H_o$ and $H_e$), and *F*-statistics were calculated with GenAlEx Version 6.5 (Peakall and Smouse 2006, 2012), and $\chi^2$ and linkage disequilibrium were estimated in GENEPOP Version 4.2 (Raymond & Rousset '95, Rousset 2008). Parental assignment was performed with Cervus (Kalinowski et al. 2007) at confidence intervals of 90 and 99%. Significance testing was performed with $\alpha$=0.05. Results presented herein represent the mean value ± SE.

*Assessment of reproductive success*

Estimates of reproductive success in typical hatchery-produced oyster families were produced from a matrix matching each progeny to the most likely male and female parents identified by Cervus. Reproductive success was then assessed in three ways. For a first crude approximation,

we calculated a weighted $N_{bwt}$ as the number of oyster parents that contributed ≥10%. We also performed an assessment of the statistical effective number of breeders, $N_{bvar}$, using the reproductive variance method from Hill (1979) as applied by Brown (2000a), where the statistical variance in reproductive success (based on the number of progeny assigned to each parent) was calculated separately for the male and female parents in each family, $\sigma^2_m$ and $\sigma^2_f$, was calculated

$$\frac{\Sigma\left(x-\bar{x}^2\right)}{(n-1)}$$

and used to calculate the effective number of breeders using the equation

$$N_b = \frac{8N_c}{(4+\sigma^2_m+\sigma^2_f)}$$

where $N_c$ was the total number of male and female parents in a family. As observed by Mewhort et al. (2009), a number of assumptions necessary for the calculation of statistical variance in a mass spawning were violated in this study, e.g., the data did not conform to a Poisson distribution and the maximum number of offspring was limited to 96. To address this concern, an approximated $N_{brand}$ for each family was calculated by performing a series of randomization tests that compared the Cervus assignments for each family to 1000 random data sets that assigned parentage evenly. Upon sequentially eliminating parents (beginning with those that were assigned zero offspring and continuing with those assigned 1, 2, 3 offspring, and so on) we recorded the number of parents that yielded a probability output that was as close as possible to "even parentage."

## RESULTS

*Genetic diversity of broodstock populations*

We observed more alleles per polymorphic locus than reported previously (Brown et al. 2000b; Reece et al. 2004; Wang and Guo 2007; Wang et al. 2009; Wang et al. 2010), ranging from A=6 at Cvi6 to A=37 at RUCV10 (Table 2, Figure 1). Linkage disequilibrium was not significant for any locus pair in the parent populations. For some loci, null (non-amplifying) alleles were apparently common. For example, the allele frequency graph for RUCV10 showed evidence of null alleles, a locus that previously had been noted to show 9/30 null alleles (Wang and Guo 2007). Family Nat1 had the most complete genotype data, with only 6.1% of the parents missing data for 3 or more loci whereas parents of Stim1 and Nat2 were missing considerably more data for 3 or more loci, 23.4% and 29.2%, respectively. However, despite differences in the numbers of parents for each family, missing data, and null alleles, the mean number of alleles observed were comparable among families $A_o$=9.7 to 12.4 as was the mean number of effective alleles per family $A_e$=5.5 to 6.4. The number of alleles with frequencies >0.05 for each locus were low, ranging from 3–9, leaving on average 71% of alleles at low frequencies. For the three parental groups, expected heterozygosities ($H_e$) ranged from 0.69–0.72 and in all cases, observed heterozygosities ($H_o$) were lower than expected by an average factor of 0.27. In each family, the allele distributions of the male and female groups differed significantly ($\chi^2$ from 57 to $\infty$, $p$<0.0001).

*Genetic diversity of the offspring populations*

Families Stim1 and Nat2 each had all 96 offspring successfully genotyped, with 4 individuals (4.1%) and 19 individuals (20.0%) missing data for 3 or more loci, respectively. Family Nat1 had 93 of the 96 offspring successfully genotyped. Of those 93, 8 individuals (8.4%) had missing data for three or more loci. For all three families, offspring exhibited greater genetic diversity and higher $H_o$ and $H_e$ than the parents (Table 2). For most of the loci in each family, a portion of the offspring showed alleles that were not found in the parents (Table 3), often peaking at lower RFU. By choosing the alleles with the highest RFU peaks, Cervus was able to assign parents to all offspring (Table 4) with 90% confidence (relaxed), and a few assignments were possible with 99% (strict) confidence (0 in Stim1, 11 in Nat1, and 6 in Nat2).

*Inbreeding levels within and among parent groups*

Inbreeding levels, $F_{IS}$ , for female parents ranged from 0.28–0.43 and for male parents from 0.26–0.41 (Table 2). Within each family, overall inbreeding estimates for the parents were $F_{IS}$ = 0.31, 0.21, and 0.40 ($n_{parents}$=24, 49, and 77 parents, respectively); thus, the level of inbreeding among the parents was between that of full-sib or parent-offspring mating (0.25) and selfing (0.5). In all three families, inbreeding of the offspring was lower than for the parent populations ($F_{IS}$ from 0.14–0.21). The level of inbreeding in the offspring populations ranged between that of half-sib (0.12) and full-sib mating (0.25).

*Effective numbers of breeders in mass spawnings*

Lower than expected reproductive success was observed for all three families, characterized by failure of 10-40% of potential parents to be assigned offspring and by considerable variation in family size for those that did contribute to the next generation. In the relatively "small" stimulated-spawning family, Stim1, where there were only 24 parents, 9 of 10 males and 13 of 14 females were assigned offspring. Conversely, in the "largest" Nat2 family, a natural spawning with 77 total parents, only 20 of 25 males and 30 of 52 females contributed to the assayed offspring. In each family, although many of the potential parents were assigned to at least one offspring, only 2–5 females and 1–3 males produced ≥10% of the offspring. Weighted estimates of effective breeder number for these three families were $N_{bwt}$ =6, 6, and 3 (for $n_{parents}$ 24, 49, and 77, respectively). For the three families examined, $\sigma^2_m$ ranged from 26 to 136 and $\sigma^2_f$ ranged from 10 to 74 (Table 5). Using the statistical estimates of variance, the average $N_{bvar}$ for the natural spawnings was 8.8 whereas the estimated $N_{bvar}$ for the stimulated-spawning was 0.9. However, because the assumptions of variance calculation were violated, these estimates were deemed unacceptable as indicators of the true reproductive success. The randomized estimates of $N_{brand}$ ranged from 7 for the smallest family to 8 for the largest family and, like the weighted estimates, were consistent with the data. Considering only the former and latter estimates of $N_b$ (Table 5), the average effective number of breeders was $N_b$ = 7.1. Comparing the $N_{bwt}$ and $N_{brand}$ values to the actual numbers of parents used in each mass spawning, the average reproductive success across all families of 0.18 indicated that only 18% of the parents actually contributed to the offspring.

## DISCUSSION

*Parentage assignment*

The number of offspring assigned to parents by Cervus was much lower at a 99% confidence than was predicted by the program's simulation of parent pairs of known sexes. Deviation from the simulated assignment power has been known to occur when other candidate parents are related to the true parents because by default, the program assumes that candidate parents are not related. Since the parents of this study had inbreeding levels indicative of full-sib or parent/offspring mating, the lower confidence of results is not surprising. Because the inbreeding level declined for offspring in all families, despite the high inbreeding levels in the parents suggests that while the females and males were very similar within their grouping, the females and males were not similar to each other. Chi-square tests and allele frequency histograms confirm this interpretation and are suggestive evidence of a Wahlund effect, whereby the spawners were comprised of two or more differentiated subpopulations. In addition to the relatively high inbreeding levels characteristic of the parent groups, the significant differences in allele frequencies among the male and female parent subpopulations also accounts for the observed reduced heterozygosity.

Difficulty in assigning parentage with the highest confidence also may have been due to the fact that some offspring exhibited alleles that were not scored in any of the candidate parents. Unexpected progeny genotypes are common in oysters, as several previous studies have noted this phenomenon in *C. virginia* as well as *C. gigas* and *O. edulis* (Wang and Guo 2007, Lallias et

al 2010, Boudry et al 2002). There are several explanations; any one or a combination of which could reasonably account for the occurrence of unexpected progeny genotypes. First, examination of Table 3 indicates that the Stim1 family showed a higher number of unexpected alleles than the two natural spawning families. This may be an indication of the unintentional contribution of sperm from the male used to induce the spawning. Another possibility is that due to the practice of scoring only the highest peaks for the two alleles, choosing the two largest peaks as the true alleles may not have been the correct approach. An alternate strategy is to pick alleles with peaks that are equal in contribution, as the extra allele of unequal contribution is likely due to locus duplication. This phenomenon has been previously attributed in oysters to the presence of both fixed and segregating alleles (Wang and Guo 2007). Non-matching alleles between parents and offspring also could be due to possible hatchery contamination as concluded by Reece et al. (2004) and/or a result of mild null allelism in the parents (Wang and Guo 2007). It also has been noted that oysters tend to exhibit segregation distortion (Boudry et al. 2002, Reece et al. 2004, Wang and Guo 2007), a phenomenon that also may be partially caused by null alleles.

*Stimulated versus natural spawnings*

Based on the current data, it is not possible to say whether natural vs stimulated spawning has any effect on reproductive success. To better assess this, the study should be replicated with similar numbers of parents for both spawning methods. It also would be beneficial to analyze more offsping from each family.

*Family size and the effective number of breeders in the hatchery*

The stimulated spawning, Stim1, consisted of a comparatively small number of parents (10 males and 14 females). However, of those parents, only one male and one female failed to contribute to the offspring and as a result, this family had the highest estimated reproductive success (0.27). Conversely, the largest family (a natural spawning) exhibited the lowest reproductive success (0.07). In the intermediate-sized Nat1 family (also naturally spawned), just under 75% of parents contributed to the offspring yielding estimated reproductive success 0.19, a result intermediate between the smallest and largest families. The reproductive success data suggest that $N_b$ is always less than $N_c$ and that breeding success is $\leq 25\%$. Reduced reproductive success also has been noted in *C. gigas* where it has been attributed to non-random mating as well as varying degrees of gamete quality and inviable genotypes (Boudry et al. 2002, Launey and Hedgecock 2001, Reece et al. 2004). Low reproductive success implies that the pedigrees of families are less variable than would be expected from paper records of parentage and in turn, inbreeding could be much higher in successive generations than would be expected. Such unintentional inbreeding could account for the observation that when certain oyster strains bred over many generations in one region are grown under different salinity, temperature, or disease-challenge situations, growth and survival can be lower than expected (Brown et al. 2005a, 2005b). Thus, if the goal of hatchery production of eastern oysters via mass spawning is to produce genetically diverse populations of oysters that can be successfully stocked or reared in a variety of environments, hatcheries would have greater success in maintaining genetic diversity and beneficial characteristics by performing more spawns with fewer parents and combining the progeny after allowing for fertilization and initial development.

**Table 1.** Details of 12 microsatellite loci used to genotype parents and offspring of three hatchery mass spawns of eastern oyster, *Crassostrea virginica*. $T_a$ (annealing temperature), $A_o$ (observed number of alleles), bp (expected base pair length).

| Locus | Primer | Primer sequence (5′–3′) | $T_a$ | $A_o$ | bp | Reference |
|---|---|---|---|---|---|---|
| RUCV045 | RUCV045-R | CACGACGTTGTAAAACGACTGTTTAGTCATGGCAGTGTGC | 57 | 30 | 198-263 | Wang and Guo 2007 |
| | RUCV045-F | GTGACTTCATTTTGAGCCTTTTACC | | | | |
| Cvi6a | Cvi6a-F | AATATTACCACGTGACCTGTGATGAATCCTTGTAGC | 57 | 24 | 171-232 | Brown et al. 2000b |
| | Cvi6a-R | CACGACGTTGTAAAACGACGTAAATATTGTATGTTCACTGTCCGGTCGTTGTGTTA | | | | |
| RUCV010 | RUCV010-F | GAAGTTAATATGGATCCGTGCTTGTA | 57 | 37 | 10-170 | Wang and Guo 2007 |
| | RUCV010-R | CACGACGTTGTAAAACGACTTATCTTTTGTATAGGGTGAGGGCAA | | | | |
| RUCV114 | RUCV114-F | GTGAGAAGGGATTGGAGTGC | 57 | 15 | 196-270 | Wang et al., 2009 |
| | RUCV114-R | CACGACGTTGTAAAACGACATGAAATAATGGCGATACGG | | | | |
| Cvi13 | Cvi13-R | CACGACGTTGTAAAACGACACCGGAGATGGTGGTATTTCC | 57 | 20 | 131-189 | Brown et al. 2000b |
| | Cvi13-F | GTGTTGCAAGACTTACAGAAGAAAC | | | | |
| Cvi12 | Cvi12-R | CACGACGTTGTAAAACGACGAGTGAGAATTTCTCGGGTGGGGC | 57 | 25 | 97-172 | Brown et al. 2000b |
| | Cvi12-F | ACTTTTTGTCACATTGACCATCCCATTTCA | | | | |
| Cvi7 | Cvi7-R | CACGACGTTGTAAAACGACTCGAAACCGAACCCTTCACCAG | 57 | 20 | 174-234 | Brown et al. 2000b |
| | Cvi7-F | TAGTGTATATCAGTTCAGACAGGTCTTTTAATGG | | | | |
| Cvi8 | Cvi8-F | GATATCCTAAACCTACTCCTCTTTTGCATTTTTG | 57 | 14 | 193-239 | Brown et al. 2000b |
| | Cvi8-R | CACGACGTTGTAAAACGACCTGAGCTTAGACTACAGCCCTACACCAG | | | | |
| RUCV6 | RUCV6-F | GCATGATACAAGATGGTGAGGTCGAT | 57 | 29 | 165-217 | Wang et al. 2007 |
| | RUCV6-R | CACGACGTTGTAAAACGACGATACTTACCTTATATGTAGCTCTGA | | | | |
| RUCV159 | RUCV159-R | CACGACGTTGTAAAACGACGGGCACATTGAAGTGTTGG | 57 | 12 | 248-295 | Wang et al. 2009 |
| | RUCV159-F | GAGGGGGAGAAATAGTGAAGG | | | | |
| RUCV164 | RUCV164-F | GGAAGAGTGTTTTGAATTGACG | 57 | 6 | 249-276 | Wang et al. 2009 |
| | RUCV164-R | CACGACGTTGTAAAACGACATATGTGATCCCCACACAAGG | | | | |
| RUCV199 | RUCV199-R | CACGACGTTGTAAAACGACGACATGGCCAATCATCTCC | 57 | 18 | 265-303 | Wang et al. 2009 |
| | RUCV199-F | TACCCCTTTATGTCCGTTCG | | | | |
| NA | Tag | CACGACGTTGTAAAACGAC | NA | NA | NA | Boutin-Ganache et al. 2001 |

**Table 2.** Genetic diversity of parents and offspring from three hatchery mass spawns of eastern oyster, *Crassostrea virginica*. $A_o$ (observed number of alleles), $A_e$ (effective number of alleles), $H_o$ (observed heterozygosity), $H_e$ (expected heterozygosity), $F_{IS}$ (inbreeding), SE (standard error).

| | $A_o$ | SE | $A_e$ | SE | $H_o$ | $H_e$ | $F_{IS}$ |
|---|---|---|---|---|---|---|---|
| Stim1* | | | | | | | |
| Males | 6.7 | 0.8 | 4.5 | 0.8 | 0.45 | 0.68 | 0.40 |
| Females | 7.6 | 1.4 | 5.4 | 1.1 | 0.52 | 0.69 | 0.28 |
| Offspring | 14.9 | 2.5 | 7.7 | 1.8 | 0.59 | 0.73 | 0.18 |
| Nat1 | | | | | | | |
| Males | 8.5 | 1.4 | 4.9 | 0.9 | 0.51 | 0.66 | 0.26 |
| Females | 10.9 | 1.6 | 6.1 | 1.3 | 0.49 | 0.70 | 0.31 |
| Offspring | 13.6 | 1.7 | 5.5 | 1.1 | 0.57 | 0.71 | 0.21 |
| Nat2 | | | | | | | |
| Males | 10.3 | 1.4 | 5.8 | 1.3 | 0.43 | 0.71 | 0.41 |
| Females | 12.9 | 2.1 | 7.0 | 1.6 | 0.42 | 0.73 | 0.43 |
| Offspring | 13.9 | 2.2 | 6.4 | 1.4 | 0.62 | 0.72 | 0.14 |

* stimulated spawn

**Table 3.** Number of alleles present in offspring but not parents of three hatchery mass spawns of eastern oyster, *Crassostrea virginica*.

| | Family | | |
|---|---|---|---|
| Locus | Stim1 | Nat1 | Nat2 |
| CVI12 | 7 | 1 | 4 |
| RUCV45 | 9 | 3 | 1 |
| CVI6a | 8 | 3 | 1 |
| RUCV164 | 2 | 0 | 0 |
| RUCV6 | 11 | 0 | 6 |
| RUCV199 | 5 | 3 | 0 |
| RUCV10 | 12 | 7 | 3 |
| Cvi7 | 6 | 3 | 5 |
| RUCV159 | 4 | 4 | 0 |
| Cvi8 | 7 | 5 | 2 |
| Cvi13 | 4 | 6 | 4 |
| RUCV114 | 1 | 2 | 0 |

**Table 4.** Summary of parentage assigned by Cervus for a typical hatchery family (Stim1) of eastern oyster, *Crassostrea virginica*.

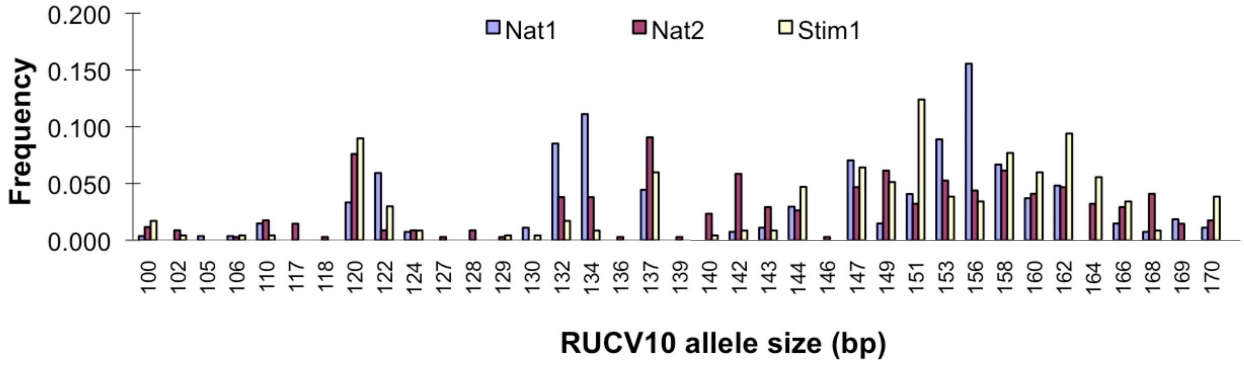
| | ♀**1** | ♀**2** | ♀**3** | ♀4 | ♀**5** | ♀**6** | ♀**7** | ♀**8** | ♀**9** | ♀**10** | ♀**11** | ♀**12** | ♀**13** | ♀**14** | **sum** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ♂**1** | | | 1 | | 3 | | 1 | 1 | | | 1 | | 1 | | 8 |
| ♂**2** | 3 | 3 | | | 16 | 2 | 2 | 3 | | 2 | 3 | 1 | | 3 | 38 |
| ♂**3** | | | | | | | | | | | | | | | 0 |
| ♂**4** | | | | | 1 | | 1 | | | 1 | 3 | | | | 6 |
| ♂**5** | | | 1 | 1 | 8 | 1 | 2 | 1 | | 2 | 4 | 1 | | | 21 |
| ♂**6** | | 1 | | 1 | 1 | | | | | | | | | | 3 |
| ♂**7** | | 1 | | | 1 | | 2 | | | | 1 | | | | 5 |
| ♂**8** | | | | | | | | | | | 1 | | | | 1 |
| ♂**9** | | | | | 1 | 1 | | 1 | | | | | | | 3 |
| ♂**10** | 1 | | 1 | | 3 | 1 | 3 | 1 | | | | | | 1 | 11 |
| **sum** | 4 | 5 | 3 | 2 | 34 | 5 | 11 | 7 | 0 | 5 | 13 | 2 | 1 | 4 | |

**Table 5.** Summary of parentage and reproductive success estimates for three hatchery spawned families of eastern oyster, *Crassostrea virginica*. Abbreviations: ≥10% (assigned to at least 10% of progeny), ≥5% (assigned to at least 5% of progeny), $\sigma^2$ statistical variance, $N_b$ (effective number of breeders), wt (weighted), var (statistical variance), rand (randomization testing). Reproductive success is $N_b$ divided by the number spawned.

| | | | | | | | Effective # of Breeders | | | Reproductive Success | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Spawned | Contributed | ≥10% | ≥0.05 | $\sigma^2$ | $N_{bwt}$ | $N_{bvar}$ | $N_{brand}$ | wt | var | rand |
| Stim1* | | | | | | | | | | | | |
| | Males | 10 | 9 | 3 | 6 | 136 | | | | | | |
| | Females | 14 | 13 | 3 | 7 | 74 | | | | | | |
| | Total | 24 | 22 | 6 | 13 | | 6 | 0.90 | 7 | 0.25 | 0.04 | 0.29 |
| Nat1 | | | | | | | | | | | | |
| | Males | 21 | 15 | 5 | 8 | 26 | | | | | | |
| | Females | 28 | 20 | 1 | 8 | 26 | | | | | | |
| | Total | 49 | 35 | 6 | 16 | | 6 | 6.94 | 13 | 0.12 | 0.14 | 0.26 |
| Nat2 | | | | | | | | | | | | |
| | Males | 25 | 20 | 2 | 6 | 47 | | | | | | |
| | Females | 52 | 30 | 1 | 7 | 10 | | | | | | |
| | Total | 77 | 50 | 3 | 13 | | 3 | 9.97 | 8 | 0.04 | 0.13 | 0.10 |

* stimulated spawn

A




B

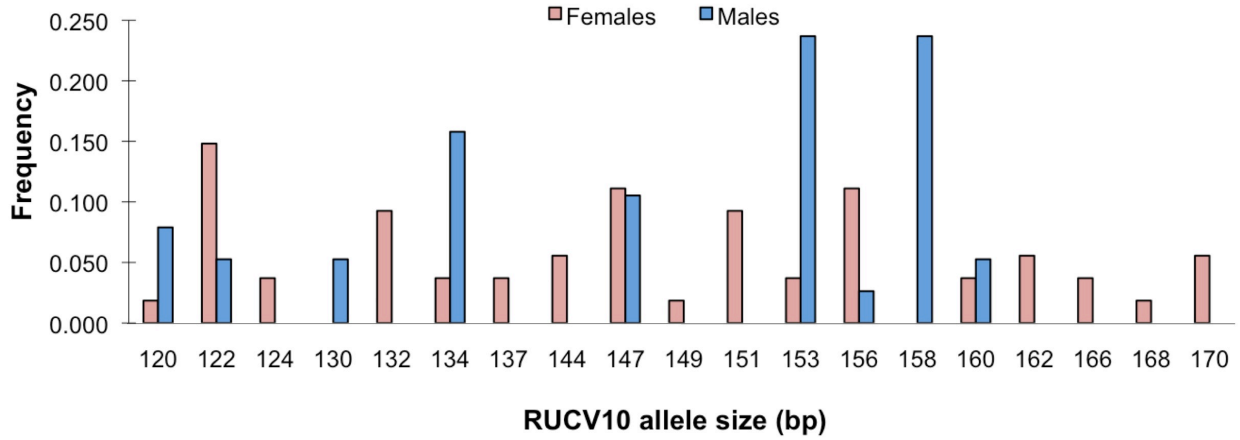



**Figure 1.** Representative allele histograms for eastern oyster, *Crassostrea virginica*, genotyped with microsatellite RUCV10. A: This locus had the highest number of observed alleles and exhibited evidence of null allelism (e.g., allele 146), possible evidence of duplication (alleles 100-139), and/or a Wahlund effect. B: Allele frequencies for the male and female parents of population Nat1, which were found to be significantly different ($p<0.0001$), as were the parent populations of the other two families.

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## APPENDIX A

### *R-script for oyster parent-progeny randomization test*

```
# Read in the file in CSV format.
oyster1 <- read.csv( file.choose(), header = TRUE )
# Get the data versus parent names
oyster2 <- oyster1 [ , 2:( ncol( oyster1 ) ) ]
# Convert all NA's to 0
oyster2[ is.na( oyster2 ) ] <- 0
# Set the number of randomization runs
n.rand1 <- 1000
# Create the output container and run the randomization runs
total.n1 <- sum( oyster2 )
total.f1 <- ncol( oyster2 )
total.m1 <- nrow( oyster2 )
Ne.out1 <- rep( 0 , n.rand1 )
for( i in 1:n.rand1 ){
  # Create a blank table
  temp1 <- matrix( 0, nrow = total.m1, ncol = total.f1 )
  # Generate the equally likely parentages (independent)
  for( j in 1:total.n1 ){
    f1.temp <- sample( 1:total.f1, size = 1 )
    m1.temp <- sample( 1:total.m1, size = 1 )
    temp1[ m1.temp, f1.temp ] <- temp1[ m1.temp, f1.temp ] + 1
  }
  f1.marg <- apply( temp1, 1, sum )
  m1.marg <- apply( temp1, 2, sum )
  vf1 <- var( f1.marg )
  vm1 <- var( m1.marg )
  Ne.temp1 <- 8*( total.f1 + total.m1 )/( 4 + vf1 + vm1 )
  Ne.out1[ i ] <- Ne.temp1
}
# Run the analysis on the data
f1.marg <- apply( oyster2, 1, sum )
m1.marg <- apply( oyster2, 2, sum )
vf1 <- var( f1.marg )
vm1 <- var( m1.marg )
Ne.actual1 <- 8*( total.f1 + total.m1 )/( 4 + vf1 + vm1 )
# P-value testing H_0: "even parentage"
mean( ifelse( Ne.out1 < Ne.actual1, 1, 0 ) )
```